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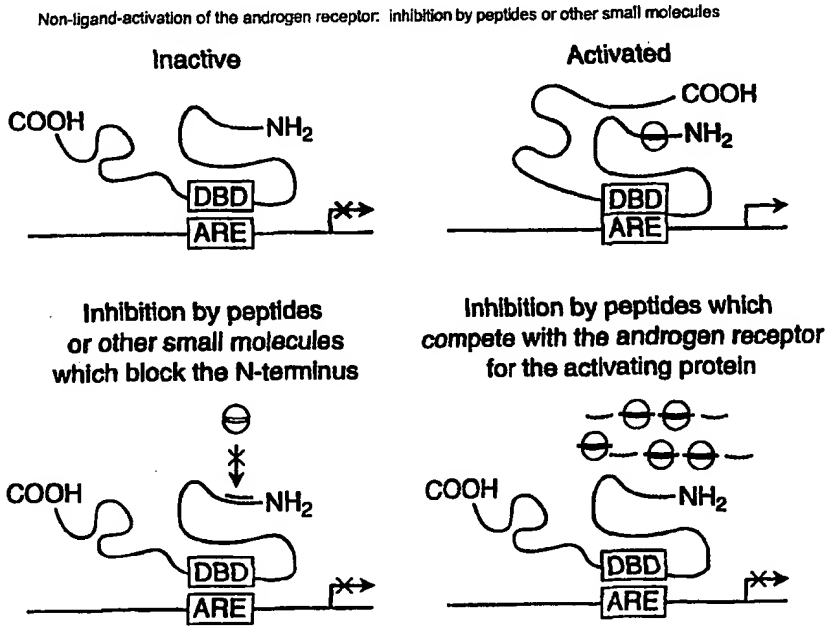

(57) Abstract

Androgen-independent activation of the androgen receptor is localized to the region of amino acids 234-391 of human androgen receptor protein. Peptides derived and nucleic acids encoding such peptides are provided. The peptides are useful as activation domains, as inhibitors of androgen-independent activation of androgen receptor, for screening compounds that affect androgen-independent activation of androgen receptor, and for treatment of androgen mediated diseases such as prostate cancer. Methods for screening compounds affecting androgen-independent activation of androgen receptor are also provided.

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INHIBITORS OF ANDROGEN-INDEPENDENT ACTIVATION OF ANDROGEN RECEPTOR

BACKGROUND OF THE INVENTION

Various diseases have an androgen mediated aspect, including prostate cancer, benign prostatic hyperplasia, hirsutism, androgenic alopecia, acne, breast cancer and Kennedy disease.

Androgen deprivation, aimed at inducing the death of androgen-dependent tumor cells is a potential treatment for such "androgen mediated diseases" and is the only useful systemic therapy available for prostate cancer in humans. The treatment involves surgical castration of the patient or treatment with anti-androgen drugs or drugs that inhibit production of androgens. The inability of androgen deprivation to eliminate all prostate cancer cell populations in a patient is manifested by a predictable pattern of response to the treatment. Initial therapy-induced regression of the malignancy is usually followed by a relapse with ultimate progression to complete resistance to further hormonal manipulations. This progression is the result of adaptation of surviving tumor cells to diminishing levels of androgen or outgrowth of subpopulations of androgen-independent tumor cells. Androgen independence appears to involve changes in androgen receptor regulated gene transcription.

The androgen receptor (AR) as a transcription factor forms the basis of a signal transduction pathway that regulates the expression of certain androgen-responsive genes. Control of androgen responsive gene activity may be agonistic (stimulated) or antagonistic (repressed) and results from binding of androgen-activated AR to upstream flanking nucleotide sequences, termed androgen response elements (AREs). The AR is activated (transformed) by androgens, including the naturally occurring androgenic hormones testosterone and dihydrotestosterone, as well as synthetic androgens such as R1881.

AR from various mammalian sources has been isolated and characterized. As well, various assays for AR are known. The deduced amino acid sequence of human AR has been reported (1) as have DNA sequences encoding human and rat AR and cells transformed with such sequences (2, 3 10).

Androgen activation of AR is mediated by ligand (androgen) binding to a ligand binding domain (LBD) located in the carboxy-terminus of the AR. In human AR, the LBD is in the region of amino acids 671-919. Certain anti-androgen compounds are also known to bind to the LBD. Androgen induced activation of the AR is associated with conformation of the AR becoming more compact upon ligand binding, heatshock proteins being dissociated, and dimerization and phosphorylation occurring prior to DNA-binding.

The DNA-binding domain (DBD) is centrally located in AR. In human AR, the DBD is in the region of amino acids 557-622. The N-terminus region preceding the DBD contributes to DNA-binding as well as transcriptional activation or repression (11). Expression of various N-terminal AR deletion and insertion mutants lacking a LBD but comprising the AR DBD as well as co-expression of various AR N-terminal fragments fused to a heterologous DBD (GAL4) with a GAL4 responsive reporter, demonstrates the presence of transcription activation domains in the first 485 amino acids and in the region of amino acids 360-528 of human AR (12).

AR belongs to the superfamily of nuclear receptors that mediate actions of lipophilic ligands, including steroids, retinoids, vitamin D3, and thyroid hormones. These receptors have distinct functional domains that include a carboxy-terminal LBD, a highly conserved DBD comprised of two zinc finger motifs, and a poorly conserved amino-terminal domain that may contain several transcriptional activation or repression domains. In all cases, binding of ligand to the receptor results in activation, such that the receptor can effectively bind to its specific DNA element.

Estrogen and progesterone receptors belong to the same family of ligand-activated transcription factors as the AR. The former receptors can be activated in the absence of their respective ligands. The estrogen receptor, for example, can be

activated through three different signaling events, i.e. not only by estrogens, but also by cAMP or by a growth factor (e.g., epidermal growth factor), each of these compounds acting via a different domain of the receptor. The AR can also be activated in the absence of androgen by elevation of cyclic AMP (cAMP) levels and by growth factors (4-6, 8). Phosphorylation has been implicated in the ligand-independent activation of the progesterone, estrogen, and retinoic acid receptors. While there are three identified phosphorylation sites on the AR, phosphorylation does not appear to be essential for the induction of androgen-regulated genes (7) and phosphorylation does not alter activity.

Androgen-independent activation of the AR involving cAMP-dependent protein kinase *A* (PKA) signal transduction pathways has been observed (6). One of the inventors named herein has determined that exposing prostate cells to forskolin (an activator of PKA) in the absence of androgen, leads to activation of the promoter of the prostate specific antigen (PSA) gene, a process shown to be mediated by the AR (9).

PSA is a clinically important androgen-stimulated gene which is used to monitor treatment responses, prognosis and progression in patients with prostate cancer. Expression of PSA is initially androgen-regulated and undergoes a sharp decline following medical or surgical castration. When the tumor becomes androgen-independent, PSA mRNA is constitutively up-regulated (9). The promoter and enhancer regions of the PSA gene have been sequenced as far as -5824 from the start site of transcription and the following DNA response elements have been characterized: TATA box , -28 to –23; androgen response elements (AREs), -170 to -156, -4148 to -4134; and, androgen response region (ARR), -395 to -376.

SUMMARY OF THE INVENTION

The inventors have found that interaction of the AR with the PKA pathway involves a discrete region of the N-terminal domain of the AR (amino acid region 234-391). This region is also involved in androgen-independent activation of the AR

resulting from treatment with a differentiating agent (butyrate). This invention now provides the means for generation of small molecules (including peptides and peptide mimetics) for inhibiting androgen-independent activation of the human AR. Such inhibitory compounds, used in combination with androgen deprivation would more effectively limit androgen mediated disease progression.

This invention provides a non-androgen ligand binding peptide comprising one or more tracts of amino acids derived from at least 10 contiguous amino acids of amino acids 234-391 of human AR, providing that said peptide does not contain a DNA-binding domain.

Peptides of this invention will comprise one or more tracts of amino acids derived from preferably at least 20, and more preferably at least 30, even more preferably at least 50 and even more preferably at least 100 contiguous amino acids derived from amino acids 234-391 of human AR. The tract of amino acids may be substantially identical to amino acids 234-391 of human AR. Peptides of this invention may comprise AR derived amino acids substantially consisting of only the aforesaid tracts of amino acids.

Peptides of this invention may substantially consist of the aforesaid tracts of amino acids or may comprise additional amino acids. Where a peptide comprises AR derived amino acids substantially consisting of one or more of the aforesaid tracts, the peptide may additionally comprise a DNA-binding domain.

This invention also provides nucleic acid constructs encoding peptides of this invention as well as said constructs in an expression vector. This invention also provides cells and pharmaceutical compositions comprising the peptides, nucleic acid constructs and expression vectors of this invention.

This invention also provides a method of inhibiting androgen-independent activation of AR by introducing into said cell, a peptide, nucleic acid construct or expression vector of this invention.

This invention also provides the use of a peptide, nucleic acid construct, expression vector or cell of this invention for the preparation of a medicament for the treatment of androgen mediated diseases including prostate tumors. This invention

also provides the use of a peptide, nucleic acid construct, expression vector or pharmaceutical composition of this invention for treatment of androgen mediated diseases including prostate tumors, particularly in patients undergoing androgen deprivation therapy.

This invention also provides a method of determining whether a compound or a mixture of compounds affects androgen-independent activation of androgen receptor (AR) comprising the steps of:

(1) contacting a compound or a mixture of compounds with a peptide comprising one or more tracts of amino acids derived from at least 10 contiguous amino acids of amino acids 234-391 of human AR; and,

(2) detecting whether a compound of said compound or mixture of compounds binds to said one or more tracts, or whether said compound or mixture of compounds affects a transcription activation or repression function of said peptide, with the proviso that when said detecting is of an effect on transcription activation or repression, said peptide will not comprise a ligand binding domain for androgen activation of AR and will comprise a DNA-binding domain.

In the preceding method, said one or more tracts of amino acids may be derived from differing lengths of contiguous amino acids of amino acids 234-391, as described above for the peptides of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic showing a general scheme for preparation of a reporter, as well as expression vectors encoding AR proteins fused to the GAL4 DBD.

FIG. 2 is a graph showing that the amino-terminus of the AR is activated by forskolin as manifested by Gal4-luciferase activity. Transactivation assays were

performed in LNCaP cells transfected with a 5xGal4UAS-TATA-luciferase reporter, Gal4DBD, and AR_N-Gal4 DBD and exposed to R1881 or forskolin (aPKA). Lanes: 1-2, control (DMSO, 0.5%); 3-4, R1881 (10 nM); 5-6, forskolin (50 μM); 1, 3, and 5, Gal4 DBD (0.5 μg); 2, 4, and 6, AR_{1-559}-Gal4 DBD (0.5 μg); 1-6, 5xGal4UAS-TATA-luciferase reporter (1.0 μg).

FIG. 3 is a schematic showing chimeric expression vectors consisting of GAL4-DBD DNA sequences fused to DNA sequences encoding various sections of the N-terminal domain of AR.

FIG. 4 is a graph showing GAL4 luciferase activity in cells transfected with the expression vectors shown in Fig. 3 and a plasmid containing the reporter shown in Fig. 1. The cells were exposed to the PKA catalytic domain (A) and butyrate (B).

FIG. 5 is a schematic showing identification of a protein(s) interacting with the 234-391 amino acid region of the AR during androgen-independent activation of the human AR.

FIG. 6 is a schematic showing two ways by which small molecules inhibit the interaction of the 234-391 amino acid region of AR with activating agents involved in androgen-independent activation of AR.

FIG. 7 is a graph showing inhibition of forskolin (FSK) mediated stimulation of PSA luciferase activity by a vector expressing a N-terminal domain of AR.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The amino acid sequence of the human AR, as determined by Lubahn *et al.* (1), is given below. The section of the N-terminal domain, (amino acids 234-391; SEQ ID NO:1) which mediates androgen-independent activation of the AR, is printed in bold letters.

1 mevqlglgrv yprppsktyr gafqnlfqsv reviqnpgpr hpeaasaapp gasllllqqq
61 qqqqqqqqqq qqqqqqqqet sprqqqqqqg edgspqahrr gptgylvlde eqqpsqpqsa
121 lechpergcv pepgaavaas kglpqqlpap pdeddsaaps tlsllgptfp glsscsadlk
181 dilseastmq llqqqqqeav segsssgrar easgaptssk dnylggtsti sdn**akelcka**
241 **vsvsmglgve alehlspgeq lrgdcmyapl lgvppavrpt pcaplaeckg slldd sagks**
301 **tedtaeyspf kggytkgleg eslgcsgsaa agssgtlelp stlslyksga ldeaaayqsr**
361 **dyynfplala gpppppppph phariklenp l**dygsawaaa aaqcrygdla slhgagaagp
421 gsgspsaaas sswhtlftae egqlygpcgg gggggggggg gggggggggg ggeagavapy
481 gytrppqgla gqesdftapd vwypggmvsr vpypsptcvk semgpwmdsy sgpygdmrle
541 tardhvlpid yyfppqktcl icgdeasgch ygaltcgsck vffkraaegk qkylcasrnd
601 ctidkfrrkn cpscrlrkcy eagmtlgark lkklgnlklq eegeasstts pteettqklt
661 vshiegyecq piflnvleai epgvvcaghd nnqpdsfaal lsslnelger qlvhvvkwak
721 alpgfrnlhv ddqmaviqys wmglmvfamg wrsftnvnsr mlyfapdlvf neyrmhksrm
781 ysqcvrmrhl sqefgwlqit pqeflcmkal llfsiipvdg lknqkffdel rmnyikeldr
841 iiackrknpt scsrrfyqlt klldsvqpia relhqftfdl likshmvsvd fpemmaeiis
901 vqvpkilsgk vkpiyfhtq (SEQ ID NO:2).

The following definitions apply to terms used in this specification.

The term "non-androgen ligand binding peptide" means a peptide capable of binding to a ligand other than androgen.

The term "tract(s) of amino acids" is a sequence of two or more amino acids joined by peptide bonds.

The term "peptide" means a compound comprising at least two amino acids joined by peptide bonds and includes a polypeptide and a protein, and peptides that comprise non-naturally occuring amino acids and peptide linkages.

The term "DNA-binding domain" (DBD) is a tract of amino acids capable of binding to DNA in a regulatory element of a gene. Examples include the androgen receptor DBD which bind to regulatory elements of androgen sensitive genes. In the case of PSA, the DBD binds to an ARE region of the PSA promoter. The DBD of GAL4 is an example of a yeast DBD used in the commercial reporter systems in

which expression of the reporter is activated or repressed by a moiety joined to the GAL4 DBD and brought into close proximity with a regulatory element (eg. promoter) of the reporter when the DBD binds to the reporter.

The term "nucleic acid" means DNA or RNA. A nucleic acid encoding a peptide is a nucleic acid containing or corresponding to a sequence of nucleotides, which sequence when transcribed and translated (or simply translated in the case of RNA) would result in the peptide. The term is meant to include any such sequence of nucleotides which, because of the degeneracy of the nucleic acid code, will encode (or corresponds to a sequence which will encode) the peptide as described above. A nucleic acid which corresponds to a sequence encoding a peptide is a nucleic acid that is complementary to a coding sequence. Nucleic acids of this invention may include anti-sense molecules where inhibition of expression of the peptides is desired.

The term 'expression vector" is a nucleic acid construct including a plasmid, phage genome or other nucleic acid which is able to replicate in a host cell and which contains a nucleic acid sequence encoding a desired peptide product. The nucleic acid sequence encoding the peptide will be expressed in the host cell. The vector may include elements necessary for incorporation of the encoding sequence into a host cell genome and the vector will contain regulatory elements operably linked to the coding sequence to permit expression of the gene product in the host cell.

The term "derived from" with reference to an amino acid sequence means that a first sequence of amino acids is substantially identical to a reference sequence of amino acids. The term "androgen receptor derived amino acids" means those parts of a peptide consisting of at least 10 contiguous amino acids which are substantially identical to a tract of 10 or more amino acids in androgen receptor. Thus, a peptide that comprises androgen receptor derived amino acids substantially consisting of a specified tract of amino acids, is a peptide in which the only tract substantially identical to a tract of amino acids in androgen receptor is the specified tract. In the latter case, the peptide may also contain tracts of amino acids not substantially identical to tracts of amino acids in androgen receptor or the peptide may consist substantially of only of tracts substantially identical to androgen receptor.

The term "substantially identical" with reference to peptides of this invention or used in this invention refers to first and reference sequences which have at least one function of a peptide of this invention in common and having at least 75% identity (more preferably at least 90% identity) as determined by comparing positions in each sequence. Sequence identity may be determined by the BLAST algorithm described in Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410, using the published default settings. When a position in the compared sequence is occupied by the same amino acid, the molecules are considered to have shared identity at that position. The degree of identity between sequences is a function of the number of matching positions shared by the sequence.

The term "derived from" is meant to include situations where conservative substitutions, deletions or additions are made in a first sequence of amino acids relative to a reference sequence. Examples of such conservative changes are the substitution of amino acids having similar "side" or "functional" groups, preferably similar in charge and ligand reactivity; most preferably also similar in size. Thus, a tract of amino acids derived from a specified number of contiguous amino acids of a reference sequence may or may not have the same number of and exact sequence of the specified contiguous amino acids of the reference, providing the tract and the specified amino acids of the reference are substantially identical as defined herein.

Nucleic acids of this invention may be synthesized when convenient or may be produced by recombinant techniques known in the art. Nucleic acids within the scope of this invention may contain linkers, modified or unmodified restriction endonuclease sites and other sequences of nucleotides useful for cloning, expression, or purification. Nucleic acids within the scope of this invention may be incorporated in a larger sequence of nucleotides, including plasmids and vectors useful for manipulation or expression of nucleic acids.

Peptides of this invention may be synthesized when convenient by any number of known peptide synthesis techniques. Alternatively, the peptides may be expressed in any suitable host cell into which an expression vector for the peptide has been introduced. The host cell and expression vector components will typically be selected

to allow for expression of the peptide in the host. Any peptide of this invention may comprise multiple (eg. repeat) tracts of amino acids derived from SEQ ID NO:1 which may enhance the activity of the peptide.

Peptides of this invention are useful as having one or more of the following functions: an agent for inhibiting androgen dependent activation of the AR; an activation domain; and, a tool for screening compounds which affect androgen-dependent activation of AR. Examples whereby peptides of this invention are tested or used for such functions are set out below. In the former case, the peptides will typically lack an intact or functional DBD, in particular a DBD that is capable of binding to an androgen sensitive gene. In such a case, the peptide might include other portions of the AR including the LBD (which will not be effective absent a functional androgen receptor DBD). Preferably, peptides of this invention used for inhibiting androgen independent activation of AR will substantially consist of androgen receptor derived amino acids which are derived only from SEQ ID NO:1. In the case of screening for compounds that affect androgen-dependent activation of AR, peptides of this invention may include a DBD if DNA-binding is desirable in the chosen mode of screening. However, where the mode of screening involves monitoring a change in pattern of activation or repression of a gene by the peptide when affected by a test compound (thereby necessitating the inclusion of DBD on the peptide), the peptide will not have an AR LBD so as to avoid an erroneous response brought about by ligand binding to the LBD.

Methods of this invention for determining whether a compound affects androgen-dependent activation of the AR may be used to detect compounds that potentially inhibit or bring about activation of the AR. In one embodiment, this method involves determining whether a compound binds to amino acid tracts derived from SEQ ID NO:1. This may be accomplished by a variety of known methods which may also facilitate the separation and recovery of the binding compound. Detection of an apparent change in conformation or molecular weight of a peptide when bound to a compound may be carried out, for example by gradient

ultra-centrifugation or by SDS PAGE. The peptide could be labeled (for example by a fluorescent compound) to facilitate separation. Alternatively, the peptide could be immobilized to facilitate separation of binding compounds. An example of such immobilization is attachment of the peptide to a suitable activated substrate (eg. beads of a chromatography gel), or by immunological techniques. Antibodies to AR and methods of producing anti-AR antibodies have been described (2, 12).

Alternate methods of screening which are part of this invention involve monitoring a change in the function of a peptide of this invention. In such embodiments, one may take advantage of the transactivation/repression characteristics of the N-terminal region of the AR and employ methods whereby enhancement or repression of such characteristics are observed as being indicative of the presence of a compound that interact with the peptide in such a way as to affect such characteristics. For example, cells may be prepared which express a chimeric protein consisting of an AR N-terminal portion including a tract derived from SEQ ID NO:1, joined to a DBD. In this example, the DBD is a "label" as the term is used herein. Following treatment of the cell with a test compound, one observes whether the function of the chimeric protein is enhanced or inhibited with respect to activation or repression of a selected "reporter". The reporter could be an androgen sensitive gene whose product is measurable or the reporter could be part of a construct intended to produce a readily detectable signal (such as the luciferase reporter used in the examples herein). A measurable androgen sensitive gene product is PSA and methods for detecting expression of PSA immunologically or by monitoring PSA mRNA are known. In the latter case, the protein "labeled" with the DBD will either not contain an AR LBD or the cells will be deprived of androgen (the cells will not contain endogenous androgen and no androgen will be applied to the cells).

Pharmaceutical compositions of this invention will typically comprise a peptide of this invention as an active ingredient and a pharmaceutically acceptable carrier. The carrier will be one which will facilitate delivery of the peptide into afflicted cells such as those of a prostate tumor. Suitable carriers for injection or diffusion of the peptide into a tumor would include traditional vehicles such as saline,

as well as more complex formulations such as liposome formulations and nonrecombinant viral vectors such as was described by Nazareth, *et al.* (6). Further, pharmaceutical compositions of this invention may include "gene therapy" vectors such as recombinant adenovirus for delivery and replication of an expression vector capable of producing a peptide of this invention.

EXAMPLES

Role of Amino Acid Region 234-391 in Androgen-Independent Activation of the Human AR

To identify the particular region of the AR involved in androgen-independent activation, use was made of a yeast transcription factor, GAL4, which consists of two well-defined domains, i.e. an activation domain and a DNA-binding domain. Chimeric proteins were generated by substituting the activation domain of GAL4 by one of a number of sections of the N-terminal region of the human AR. While these chimeric proteins had a DNA-binding site located in their GAL4 segment, they did not have the LBD of AR.

To generate chimeric proteins, expression vectors were constructed as described below, consisting of DNA encoding the DNA-binding domain (DBD) of GAL4 (supplied by Clontech) fused to DNA encoding various regions of the AR: i.e. a region including the N-terminal region of AR [amino acids (aa) 1 to 558], and regions aa 392 to 558, aa 1 to 233, aa 234 to 391 and aa 1 to 391 (Figures 1 and 3). A luciferase reporter vector (Clontech), consisting of the luciferase gene and the GAL4 DNA response element located upstream of the luciferase gene, was used for assessing activation of transcriptional activity of the chimeric proteins. Another vector, containing the catalytic domain of PKA (Clontech), was used to induce PKA signaling. Following co-transfection of the various vectors into human prostate cancer cells and incubation with stimuli, luciferase activity in cell lysates was assayed as described below.

All chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise. PC3 cells between the 30th and 45th generation, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (GIBCO/BRL, Burlington, Ontario, Canada). LNCaP cells between the 44th and 55th generation were maintained in RPMI 1640 supplemented with 5% FBS. When the plates or wells were 60-70% confluent with cells, the culture medium was changed to serum-free medium containing vehicle (DMSO), R1881, or forskolin.

The AR_NGal4 plasmid was constructed by PCR amplification of the nucleotides 363 to 2039 of human AR cDNA using primers 5'-AAA GGA TCC GGA TGG AAG TGC AGT TAG GGC T (SEQ ID NO: 3) and 5'-AAA AGG ATC CTT CAG GTC TTC TGG GGT GGA AAG TAA TAG (SEQ ID NO:4). The amplified DNA fragment was purified, blunt-end ligated into the *Eco*RV site of Bluescript SK(-) excised with *Bam*HI and cloned into the *Bam*HI site of pFA-CMV plasmid (Stratagene Cloning System, La Jolla, CA). Similarly, constructs containing different AR N-terminal domains were prepared. The orientation and sequences were confirmed by DNA sequence analysis and expressed protein was detected by Western blotting. An expression vector for the catalytic subunit of protein kinase A (PKAc) was purchased from Stratagene (PathDetect CREB trans-Reporting System).

LNCaP cells (3×10^5) were plated on 6-well plates and incubated in RPMI 1640 with 5% FBS prior to transfection. Total amount of plasmid DNA used was typically normalized to 3 μg/well by the addition of empty plasmid. Medium was replaced after 24 h by serum-free RPMI 1640 containing variously, DMSO, R1881, or forskolin. Cells were collected after 48 h incubation.

PC3 cells (3×10^5) between the 30th and 45th generation, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (GIBCO/BRL, Burlington, Ontario, Canada). Total amount of plasmid DNA used was typically normalized to 3 μg/well by the addition of empty plasmid. Cells were transfected in serum-free conditions for 5 h and then incubated with forskolin (FSK, 50μM), R1881 (10 nM), or vehicle (DMSO, 0.5%), for an additional 16 h under serum-free conditions before harvesting.

Luciferase activities in cell lysates were measured using the Dual Luciferase Assay System (Promega, Madison, WI). Luciferase activities were normalized by the Renilla activities and protein concentrations of the samples. The results were converted to fold-induction, which is the relative luciferase activity of the treated cells over that of the control cells, or activity in the presence of a stimulus over activity absent stimuli.

Amino terminal fragments containing approximately amino acids 1-559 (AR_N) of the human AR were cloned into the carboxyl terminus of Gal4 DBD. Expression vectors for these chimeric proteins were cotransfected into LNCaP cells with the above-described reporter gene which contains the Gal4-binding site as a *cis*-acting element (p5xGal4UAS-TATA-luciferase). LNCaP cells express endogenous AR and PSA. As shown in Fig. 2, the synthetic androgen R1881 did not significantly change the activity of the reporter when comparing AR_NGal4DBD (lane 4) to Gal4DBD lacking the amino-terminus of the AR (lane 3). Upon the addition of forskolin, the Gal4 DBD fused to the N-terminal fragment of human AR activated the Gal4-luciferase reporter 60-fold (lane 6) over levels achieved with the Gal4 DBD lacking the amino-terminus of the AR (lane 5). These results show that the amino-terminal domain of the AR is targeted by the PKA pathway of androgen independent activation of AR.

Figure 4 shows that non-androgen activation of transcriptional activity of the chimeric proteins can be achieved in response to two different stimuli, i.e. PKA signaling and butyrate, a differentiating agent. Construct 1-558 (which includes the complete amino terminal region of the AR) substantially enhanced GAL4 induction of the luciferase reporter in PC3 cells. PC3 cells are of human prostate cancer origin but are poorly differentiated and do not express AR or PSA. This activation was localized to amino acid region 234-391, since only constructs containing this amino acid region (1-558, 234-391 and 1-391) showed enhanced induction. Furthermore, constructs not containing amino acid region 234-391 (i.e. 1-233 and 392-558) did not increase induction above that obtained with a construct containing only the GAL4

DNA-binding site (GAL4-DBD). Thus, non-androgen activation is confined to amino acid region 234-391 of the AR.

Identification of Proteins Interacting with Amino Acid Region 234-391 of the Human AR

Figure 5 shows an example procedure for identification of proteins interacting with amino acid region 234-391 of the AR during its androgen-independent activation. As indicated, cells are transfected with e.g., a GAL4-AR234-391 plasmid construct, and then exposed to stimuli. The cells are then lysed and the GAL4-AR fusion protein is precipitated with an antibody specific for the GAL4 segment of the chimeric protein. If, as a result of stimulation, a protein(s) binds to the expressed 234-391 region of the AR, it will be co-precipitated along with the GAL4-AR fusion protein and may be detected as an additional protein band on an SDS polyacrylamide gel. Recovery and characterization of the protein could then be done using standard procedures.

Development of Inhibitors of Androgen-Independent Activation of the Human AR

Figure 6 shows two mechanisms by which small molecules (e.g., peptides, peptide mimetics) would inhibit the interaction of the 234-391 amino acid region of the AR with activating agents (proteins). As shown in the left bottom corner, a small molecule binds to the 234-391 region of the AR and as a result, prevents activation. Alternatively, as shown in the right bottom corner and in the example below, compounds mimicking the 234-391 region of the AR (in terms of amino acid sequences or active sites) compete with the AR for the activating protein and hence prevent activation of the AR.

The first category of inhibitory compounds may be developed, for example, using a phage display method based on the binding of small peptides from a combinatorial phage library to a recombined "bait" protein, containing the 234-391 amino acid region of the human AR or sections thereof (eg. 10-20 mer sections).

Phage display protein ligand screening systems are described by Lowman, H B. *et al.*, Biochem. 30:10832-10838 (1991); Markland, W. et al., Gene 109:13-19 (1991); Roberts, B. L. et al., Proc. Natl. Acad. Sci. (U.S.A.) 89:2429-2433 (1992); Smith, G. P., Science 228:1315-1317 (1985); and, Smith, R. P. *et al.*, Science 248:1126-1128 (1990). In general, this method involves expressing a fusion protein in which a putative ligand for the "bait" is fused to the N-terminus of a viral coat protein (such as the M13 Gene III coat protein, or a lambda coat protein). A library of phage are engineered to display putative peptide ligands on the coat protein. The phage are placed in contact with the bait. Phage that display coat protein having peptides that are capable of binding to the bait are immobilized by such treatment, whereas all other phage can be washed away. After the removal of unbound phage, the bound phage can be amplified, and the DNA encoding their respective coat proteins is sequenced. In this manner, the amino acid sequence of peptides that bind to the "bait" can be deduced.

The second category of inhibitors could be developed by examining the inhibitory effect on non-ligand activation of the GAL4-AR234-391 chimeric protein of short peptides (10-20 mer length) which encode fragments of amino acid region 234-391 of the human AR. To overcome possible cellular uptake problems, potentially inhibitory peptides could be introduced into the cells in the form of expression vectors encoding the sequences of the peptides. Animal tumor models are available for *in vivo* testing of identified inhibitory compounds.

Inhibition of Androgen-Independent Activation of the Human AR with N-terminal AR Fragments

Androgen-independent AR expression of the prostate specific antigen (PSA) gene can be obtained in cultured LNCaP human prostate cancer cells by incubating the cells with forskolin, an activator of PKA (9). In this example, a luciferase reporter vector consisting of the PSA promoter located upstream of the luciferase gene, was used for assessing androgen-independent activation of the PSA gene. In order to construct the PSA promoter plasmid (PSA), PSA 5' flanking DNA (-630/+12) was

obtained by PCR amplification of human genomic DNA using oligonucleotide primers corresponding to the PSA gene and ligated with *Eco*Rv-digested pBluescript™ (pBS) sk(-) (Stratagene, La Jolla, CA, USA) according to a method previously described (10).

A chimeric protein was generated by inserting the N-terminal region [amino acids 1 to 558] of the human AR into a His-tag expression vector (In Vitrogen). The expression vector (1-558) or the vector with no N-terminal domain (HisTag) were co-transfected with the PSA-luciferase reporter into LNCaP human prostate cancer cells and incubated without androgens in the presence or absence of forskolin. As an additional control, the luciferase reporter without the PSA promoter (pGL2) was also co-transfected into LNCaP cells.

Figure 7 shows non-ligand activation of AR by forskolin (PKA stimulation) (HisTag+PSA). In the control (non-treated LNCaP cells), there is very little luciferase activity showing that the PSA promoter is not being stimulated. Upon addition of forskolin (FSK), there is a dramatic increase in luciferase activity indicating non-androgen stimulation of PSA. The empty vector (HisTag) had no effect on forskolin stimulation of PSA. Expression of the pGL2 vector does not increase upon addition of forskolin showing that activation is dependent upon the PSA promoter (HisTag+pGL2). There is a significant drop in PSA luciferase activity when HisTag is replaced with vector expressing the complete amino terminus of the AR, (compare 1-558+PSA in the presence of FSK to HisTag+PSA in the presence of FSK). The decrease in luciferase activity shows that the AR N-terminal peptide fragment is suppressing forskolin stimulation of the PSA promoter. Thus, a N-terminal fragment can inhibit androgen-independent activation of the human AR through competition.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of skill in the art in light of the teachings of this invention that changes and modification may be made thereto without departing from the spirit or scope of the appended claims. All patents, patent applications and publications referred to herein are hereby incorporated by reference.

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WE CLAIM:

1. A non-androgen ligand binding peptide comprising one or more tracts of amino acids derived from at least 10 contiguous amino acids of SEQ ID NO:1, providing that said peptide does not contain a DNA-binding domain.

2. The peptide of claim 1 wherein at least one of said one or more tracts is derived from at least 20 contiguous amino acids of SEQ ID NO:1.

3. The peptide of claim 1 wherein at least one of said one or more tracts is derived from at least 30 contiguous amino acids of SEQ ID NO:1.

4. The peptide of claim 1 at least one of said one or more tracts is derived at least 50 contiguous amino acids of SEQ ID NO:1.

5. The peptide of claim 1 at least one of said one or more tracts is derived from at least 100 contiguous amino acids of SEQ ID NO:1.

6. The peptide of claim 1 at least one of said one or more tracts is substantially identical to SEQ ID NO:1.

7. A peptide of any one of claims 1-6, wherein the peptide comprises androgen receptor derived amino acids substantially consisting only of said one or more tracts of amino acids.

8. The peptide of claim 7 joined to a DNA-binding domain other than an androgen receptor DNA-binding domain.

9. A nucleic acid encoding a peptide according to any one of claims 1-8.

10. An expression vector comprising the nucleic acid of claim 9.

11. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an active agent selected from the group consisting of: a peptide according to any one of claims 1-8; a nucleic acid of claim 9; and, an expression vector of claim 10.

12. A cell comprising a peptide according to any one of claims 1-8, a nucleic acid of claim 9, or an expression vector of claim 10.

13. A method of inhibiting androgen independent activation of androgen receptor comprising the step of introducing into an androgen receptor containing cell, an active agent selected from the group consisting of: a peptide according to any one of claims 1-8; a nucleic acid according to claim 9; and, an expression vector according to claim 10.

14. The method of claim 13 wherein said cell is deprived of androgen.

15. The use of a peptide according to any one of claims 1-8, a nucleic acid according to claim 9, an expression vector according to claim 10, or a cell of claim 12, for preparation of a medicament for treatment of androgen mediated diseases.

16. The use of a peptide according to any one of claims 1-8, a nucleic acid of claim 9, an expression vector of claim 10, or a pharmaceutical composition of claim 11, for treatment of androgen mediated diseases.

17. The use of claim 16 wherein said treatment is of a prostate tumor in a patient deprived of androgen.

18. A method of determining whether a compound or a mixture of compounds affects androgen-independent activation of androgen receptor comprising the steps of:

(1) contacting a compound or a mixture of compounds with a peptide comprising one or more tracts of amino acids derived from at least 10 contiguous amino acids of SEQ ID NO:1; and,

(2) detecting whether a compound of said compound or mixture of compounds binds to said one or more tracts, or whether said compound or mixture of compounds affects a transcription activation or repression function of said peptide, with the proviso that when said detecting is of an effect on transcription activation or repression, said peptide will not comprise a ligand binding domain for androgen activation of androgen receptor and will comprise a DNA-binding domain.

19. The method of claim 18 wherein at least one of said one or more said tracts is derived from at least 20 contiguous amino acids of SEQ ID NO:1.

20. The method of claim 18 wherein at least one of said one or more said tracts is derived from at least 30 contiguous amino acids of SEQ ID NO:1.

21. The method of claim 18 wherein at least one of said one or more said tracts is derived from at least 50 contiguous amino acids of SEQ ID NO:1.

22. The method of claim 17 wherein at least one of said one or more said tracts is derived from at least 100 contiguous amino acids of SEQ ID NO:1.

23. The method of claim 18 wherein at least one of said one or more tracts is substantially identical to SEQ ID NO:1.

24. The method of any one of claims 18-23 wherein said peptide is immobilized or labeled, and said detecting is of a compound binding to the labeled or immobilized peptide.

25. The method of any one of claims 18-23 wherein said detecting is of an effect on transcription activation or repression of a gene to which said DNA-binding domain binds.

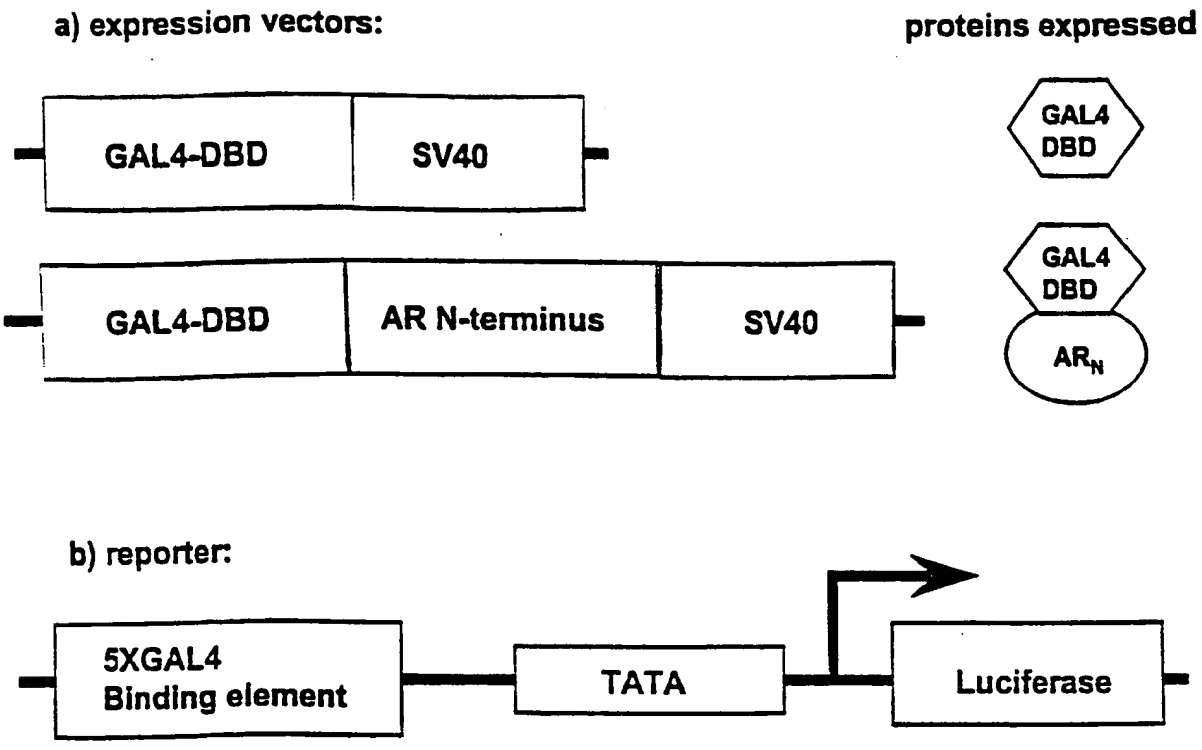

a) expression vectors:
proteins expressed
GAL4-DBD
SV40
GAL4 DBD
GAL4-DBD
AR N-terminus
SV40
GAL4 DBD
AR$_N$
b) reporter:
5XGAL4 Binding element
TATA
Luciferase

Fig. 1

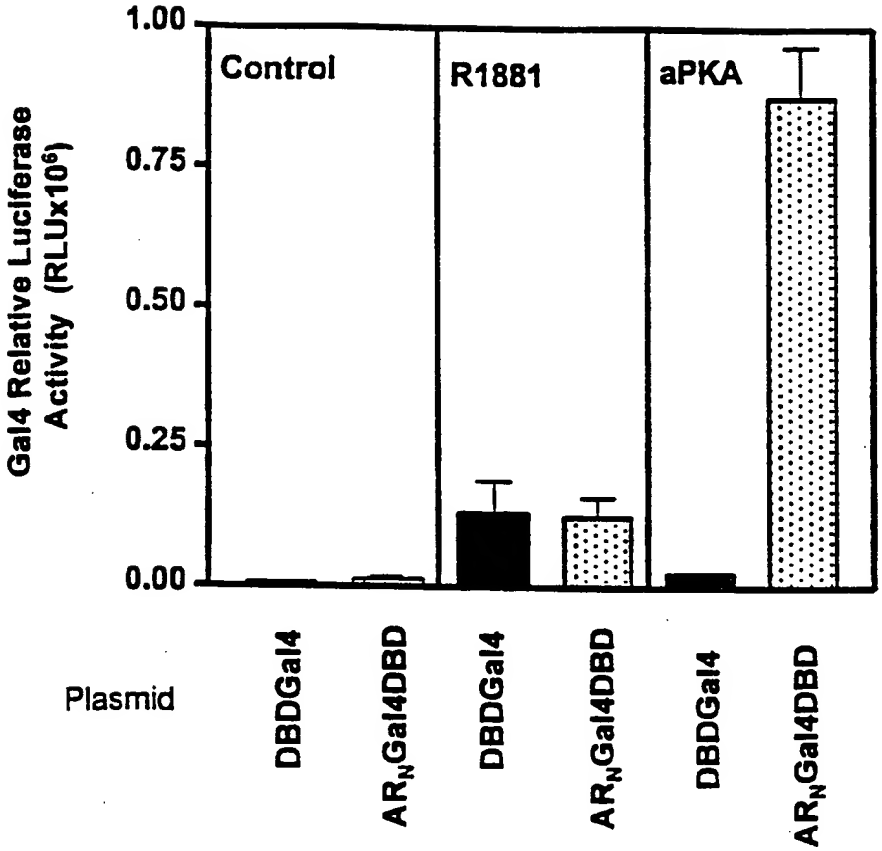

Gal4 Relative Luciferase Activity (RLUx10⁶)
1.00
0.75
0.50
0.25
0.00
Control
R1881
aPKA
Plasmid
DBDGal4
AR_NGal4DBD
DBDGal4
AR_NGal4DBD
DBDGal4
AR_NGal4DBD

Fig. 2

GAL4-Human AR constructs

GAL4DBD / human N-terminal AR domain

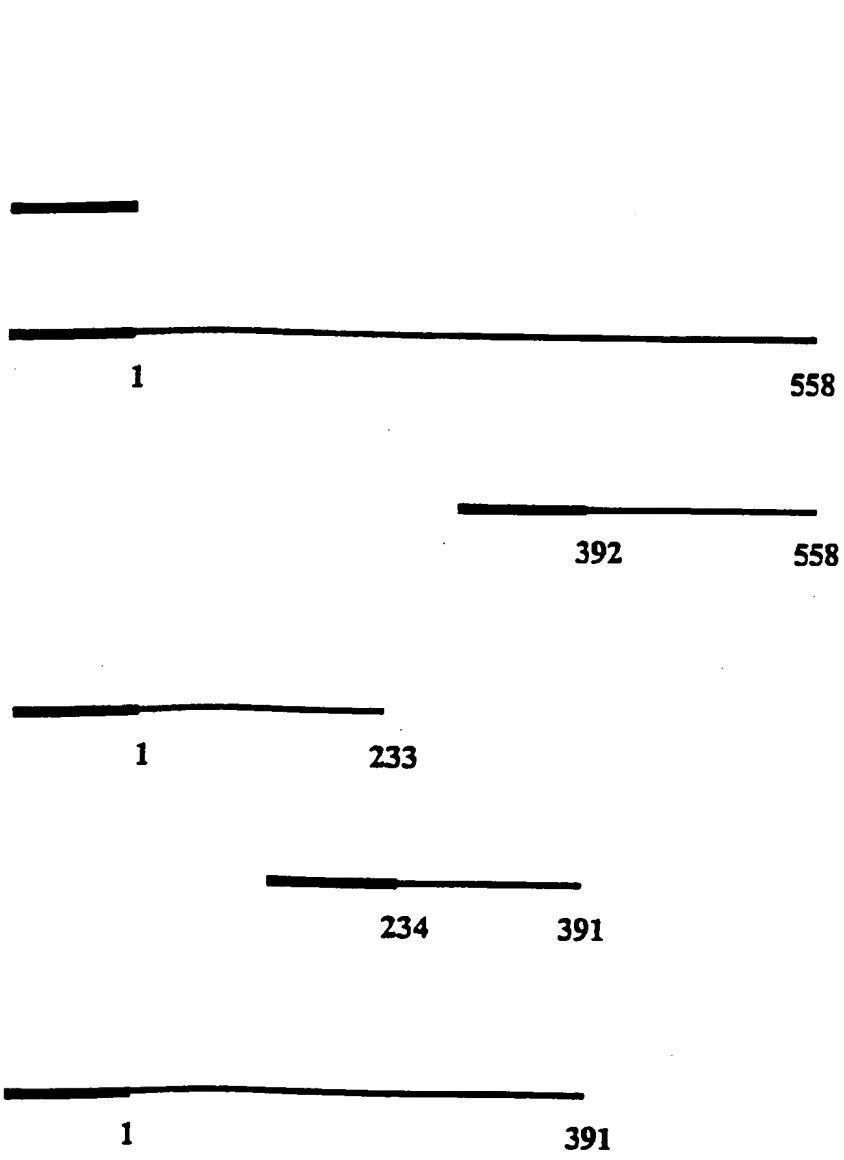
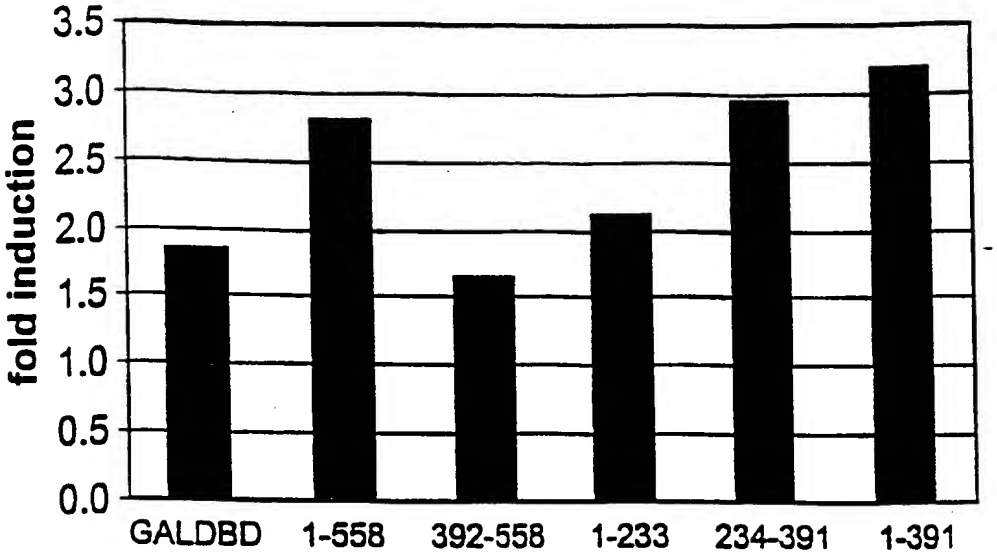


Fig. 3

A


fold induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
GALDBD
1-558
392-558
1-233
234-391
1-391

B

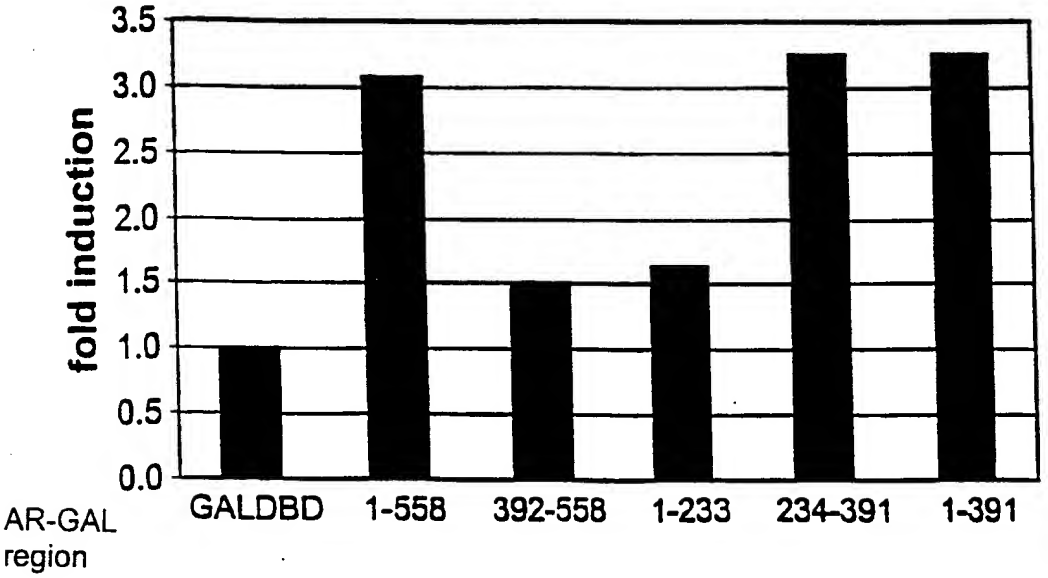
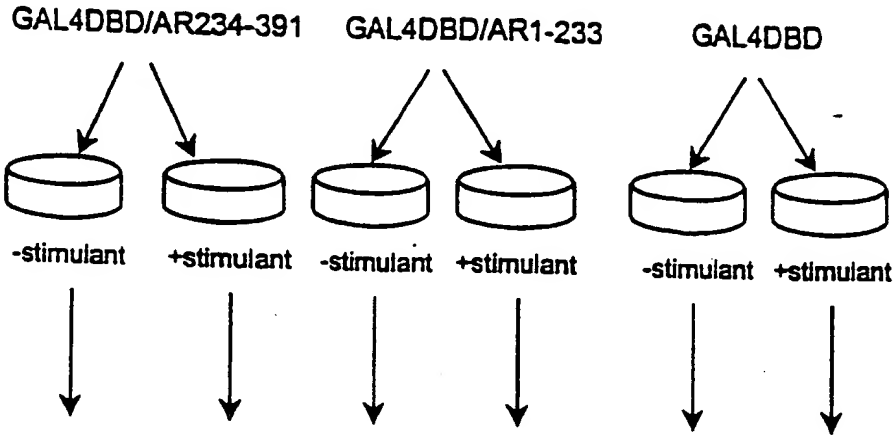

fold induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
GALDBD
1-558
392-558
1-233
234-391
1-391
AR-GAL region

Fig. 4

Transfection of cells with expression vectors encoding GAL4DBD fused to the following AR amino acid regions:



Immunoprecipitation of proteins interacting with the expressed chimeras using antibodies to the GAL4DBD, then followed by SDS-PAGE

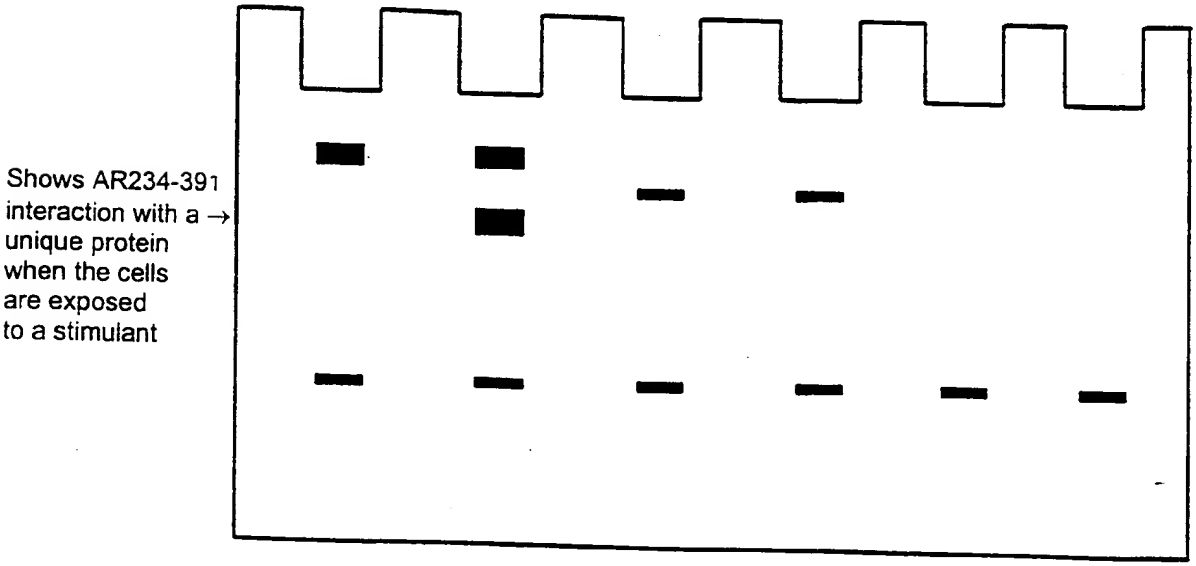


Fig. 5

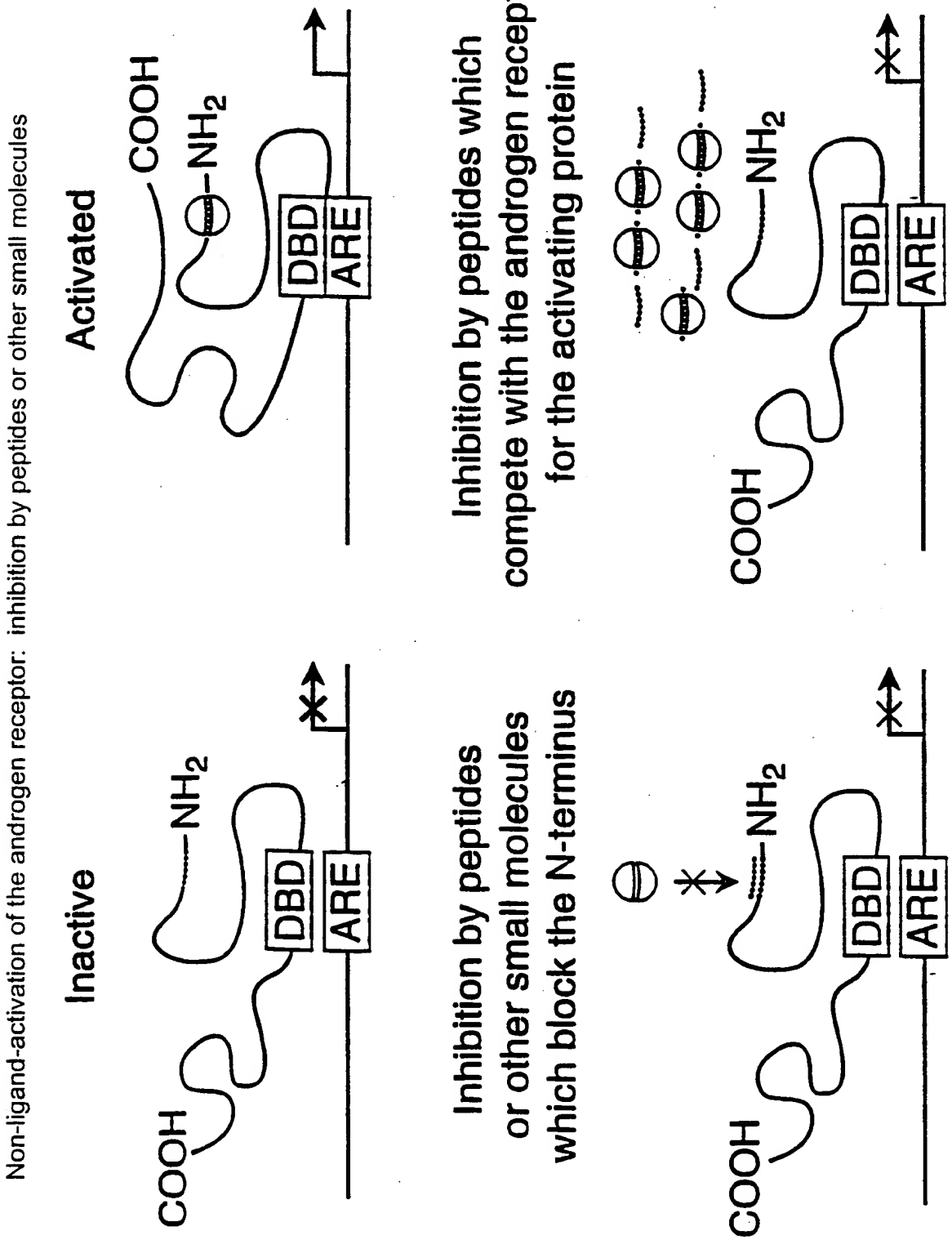
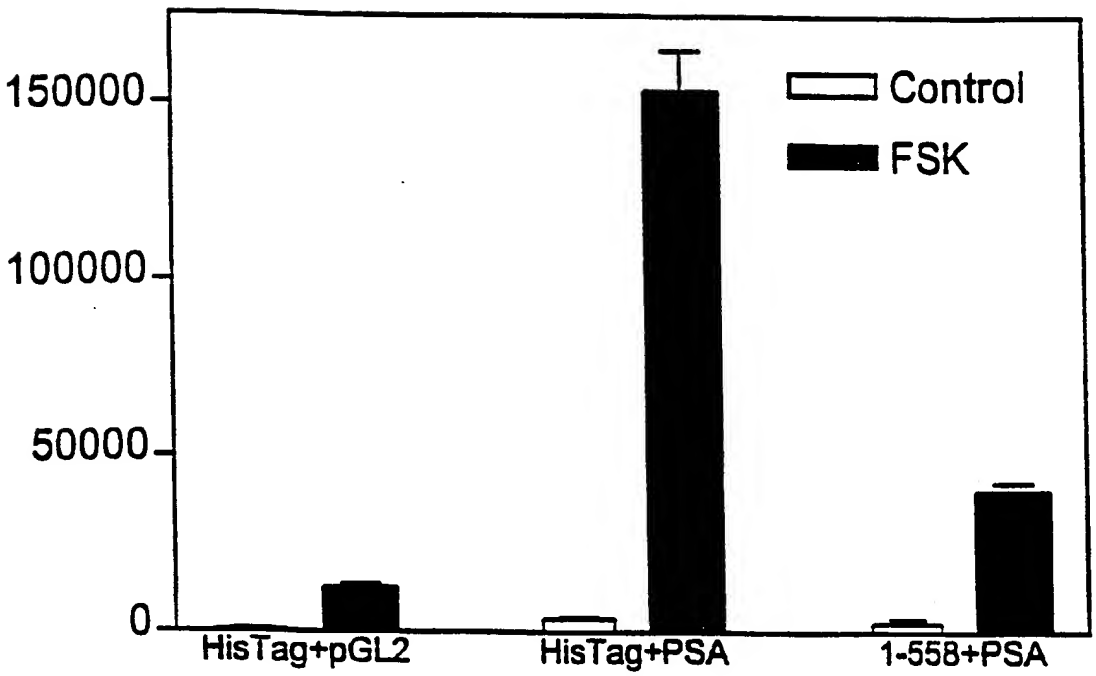

Non-ligand-activation of the androgen receptor: inhibition by peptides or other small molecules
Inactive
Activated
COOH
NH2
DBD
ARE
Inhibition by peptides or other small molecules which block the N-terminus
Inhibition by peptides which compete with the androgen receptor for the activating protein

Fig. 6



Plasmids transfected into LNCaP cells

Fig. 7

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A3

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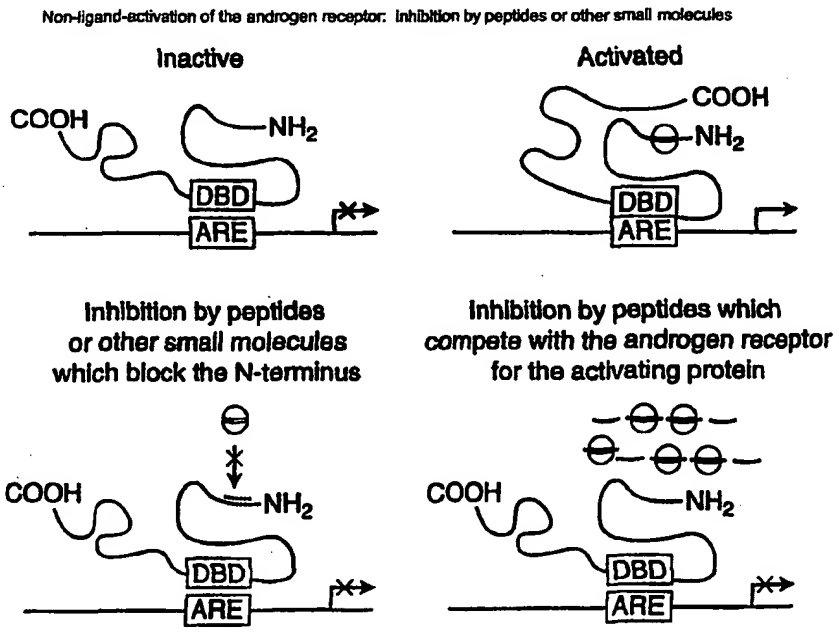

(57) Abstract

Androgen–independent activation of the androgen receptor is localized to the region of amino acids 234–391 of human androgen receptor protein. Peptides derived and nucleic acids encoding such peptides are provided. The peptides are useful as activation domains, as inhibitors of androgen–independent activation of androgen receptor, for screening compounds that affect androgen–independent activation of androgen receptor, and for treatment of androgen mediated diseases such as prostate cancer. Methods for screening compounds affecting androgen–independent activation of androgen receptor are also provided.

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 99/00604

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/72 A61K38/17 C12N5/10

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Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 89 09791 A (UNIV NORTH CAROLINA) 19 October 1989 (1989-10-19) cited in the application page 8, line 5-32 page 21, line 18-30 page 24, line 1-3 SEQ.ID.N.1	1-7,9,10

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A	NAZARETH LV AND WEIGEL NL: "Activation of human androgen receptor through a protein kinase A signaling pathway" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, 1996, pages 19900-19907, XP002130095 cited in the application page 19900 -page 19901, left-hand column, line 1-20	1,11-18
A	JIA L D ET AL: "Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells" STEROIDS: STRUCTURE, FUNCTION, AND REGULATION,US,ELSEVIER SCIENCE PUBLISHERS, NEW YORK, NY, vol. 61, no. 9, 1996, page 531-539 XP004070285 ISSN: 0039-128X	1,10,12, 14,18,24
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INTERNATIONAL SEARCH REPORT

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Inter nal Application No
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WO 9730074 A	21-08-1997	AU 2272397 A	02-09-1997
		CA 2246378 A	21-08-1997
		EP 0897392 A	24-02-1999
WO 8909791 A	19-10-1989	AT 182901 T	15-08-1999
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